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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EU971892549US

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☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

Diagnostic and Therapeutic Applications of SED1

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 19 ☐ CD(s), Number _____

☐ Drawing(s) *Number of Sheets* _____ ☐ Other (specify) _____

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

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Respectfully Submitted,

SIGNATURE _____

Date 10/17/03

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REGISTRATION NO. *(if appropriate)* 44,917

TELEPHONE 404-727-5256

Docket Number: 03093Prov

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Provisional Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Re: **U.S. Provisional Patent Application**
Filed: October 17, 2003
For: "Diagnostic and Therapeutic Applications of SED1"
Emory File No.: 03093Prov

Dear Sir:

Enclosed for filing regarding the above-referenced patent application are:

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Helen G. Carter _____ Date _____

TITLE OF INVENTION

Diagnostic and Therapeutic Applications of SED1

CROSS-REFERENCE TO RELATED APPLICATIONS

Not Applicable

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Support for research leading to this invention was provided in part by the National Institutes of Health Grant No. HD23479. The United States Government has certain rights with respect to the invention.

BACKGROUND

About 15% of couples have reduced fertility and approximately one-half of these are due to male infertility, usually of genetic origin. Common treatments include drug therapy, surgery, testicular sperm extraction (TESE) and magnelaser therapy. Genetic defects are believed to be the most prevalent cause of abnormalities, which result in the loss of specific DNA segments and ultimately leads to loss of vital genes for sperm production.

There are several technologies currently under development that target male infertility, including recombinant human zona pellucida protein (rhZP3) and glycosylated peptides having biological activity of binding to human spermatozoa, a TIAP polypeptide, and use of n-acetyl-l-cysteine to treat and prevent inflammation-based infertility.

Addressing male contraception is a much trickier subject since men produce approximately 100 million sperm every day, compared to women, who produce one functional gamete per month. That having been said, there are numerous ongoing efforts aimed at male contraception.

The invention described herein represents a major step forward in understanding the cell-cell interactions that occur during a crucial step in mammalian fertilization. In order for a sperm to fertilize an egg, it must bind to the egg's outer layer. This attachment is highly specific, as sperm generally only recognize eggs from the same species. The protein described here, SED1, is involved in this attachment. The absence of SED1 significantly decreases fertility. SED1 can be used as the technological basis for infertility treatments and/or contraceptive treatments. The invention also provides the technology basis for diagnosis of infertility.

BRIEF SUMMARY OF INVENTION

The invention described herein may be used for both diagnostic and therapeutic purposes. The invention describes the SED1 protein.

Mouse SED1 is located on the surface of mouse sperm. Antibodies against SED1 block sperm-egg binding. Genetically engineered mice that lack SED1 were significantly less fertile than normal mice. Although the sperm from SED1-deficient mice were mobile and produced in normal quantities, the sperm generally failed to bind eggs, proof that SED1 is an integral component of sperm-egg binding.

The invention described herein comprises diagnostic tests, and methods to diagnose, the presence or absence of SED1 in mammalian species. The invention also contemplates diagnostic tests and methods to assay for mutant SED1 proteins, as well as methods and tests to quantify the protein. The various versions of SED1 across different mammalian species have substantial homology. Human SED1 is also known in the literature as BA46.

In another aspect of this invention, the invention comprises compositions of SED1, homologous proteins (including truncations, deletions, etc.), and peptides derived from SED1, useful for enhancing fertility by adding to sperm or eggs in vitro or in vivo. The invention also contemplates compositions of peptides, peptide mimetics, and small molecules that resemble the binding motifs in SED1. Said compositions can be applied in any manner such that they may bind to sperm or eggs, such as, for example, topical application of a vaginal cream containing said composition. For example, SED1 homologues could be incorporated into commercially available vaginal moisturizers. Alternatively, SED1 homologues could be added to *in vitro* mixtures of sperm and eggs. This process would greatly facilitate conception for couples attempting in vitro fertilization wherein a diagnostic test had indicated that the sperm lacked sufficient, functional SED1-type protein. Relative molar ratios of SED1 or SED1 variants to sperm could be between 0.1 to 1 and one billion to one.

The invention comprises methods of producing recombinant SED1 and SED1 variants. Post-translational processing does not appear to significantly affect the protein's functional ability, at least as it relates to sperm-egg binding. Accordingly, the invention specifically contemplates methods and composition for producing SED1 and SED1 variants (including deletions, mutations, and truncations, and combinations thereof, wherein the SED1 variants maintains at least 80% of the binding affinity of the native protein) in bacteria (e.g., *Bacillus subtilis*) and yeast (e.g., *Saccharomyces cerevisiae*) species.

In another aspect of this invention, SED1 could be the basis for treatments aimed not at increasing the likelihood of fertilization, but rather targeted at preventing it. This application describes experimental results showing that sperm-egg binding can be disrupted by molecules that block the binding site. Accordingly, this invention comprises methods for preventing pregnancy comprising administering SED1, peptides thereof, or mimetics thereof as competitive inhibitors of the binding site on eggs that is targeted by SED1 and related proteins.

The invention also describes antibodies raised against SED1 or SED1 mimetics, wherein the antibodies could inhibit fertilization, when applied topically. The invention also contemplates the use of SED1 or SED1 mimetics as immunogens sufficient to generate an immune response that can aid in preventing pregnancy.

The invention describes methods for increasing fertilization, or preventing fertilization, in humans as well as other mammalian species.

DETAILED DESCRIPTION OF INVENTION

Pharmaceutical compositions of the invention described herein can incorporate any of the techniques, adjuvants, and excipients known in the art. Physiologically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., Remington's Pharmaceutical Sciences, 17.sup.th ed., 1989). Administration can be in any convenient manner, e.g., by injection, oral administration, inhalation, transdermal application, or rectal administration, although topical and intra-vaginal administration are particularly contemplated. Dosages and administration may be determined using the in vivo and in vitro models provided herein.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the invention will be described in greater detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art

in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Diagnostic tests for determining the presence or absence of SED1, or quantification of that protein, may utilize any methods known in the art.

Biochemical and computational methods may be used to identify binding motifs on SED 1 for binding to both the sperm and egg. In particular, modeling methods may be used to identify potential competitive inhibitors of these binding sites.

Definitions:

"SED1 Functional Protein" shall mean SED1 or SED1 variants, including truncation, deletion, and mutation sequences, and combinations thereof, wherein the protein has the ability to bind both sperm and egg with at least 50% of the affinity of the native species.

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Identification of Mouse Sperm SED1, a Bimotif EGF Repeat and Discoidin-Domain Protein Involved in Sperm-Egg Binding

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Summary

We report the identification of SED1, a protein required for mouse sperm binding to the egg zona pellucida. SED1 is homologous to a small group of secreted cell-matrix adhesive proteins that contain Notch-like *EGF* repeats and *d*iscoidin/F5/8 type C domains. SED1 is expressed in spermatogenic cells and is secreted by the initial segment of the caput epididymis, resulting in SED1 localization on the sperm plasma membrane overlying the acrosome. SED1 binds specifically to the zona pellucida of unfertilized oocytes, but not to the zona of fertilized eggs. Recombinant SED1 and anti-SED1 antibodies competitively inhibit sperm-egg binding, as do truncated SED1 proteins containing a discoidin/C domain. SED1 null males are subfertile and their sperm are unable to bind to the egg coat in vitro. These studies illustrate that Notch-like EGF and discoidin/C domains, protein motifs that facilitate a variety of cellular interactions, participate in gamete recognition as well.

Introduction

Successful fertilization in mammals is dependent upon the species-specific recognition, adhesion, and fusion between sperm and egg. Despite their fundamental importance, we still know little about the molecular basis underlying these events. Two sperm-egg recognition events in particular have received the most attention: the initial adhesion between the sperm plasma membrane and the egg extracellular coat, or zona pellucida, and the binding between membranes of the acrosome-reacted sperm and the egg plasma membrane (Primakoff and Myles, 2002; Wassarman et al., 2001). In both instances, candidate receptors have been identified, but thus far, none of these receptors appear to be completely responsible for either sperm-egg binding or sperm-egg fusion (Miller et al., 1992; Nishimura et al., 2001; Rankin et al., 1998).

In particular, sperm binding to the zona pellucida is thought to involve recognition of specific glycoside residues on the ZP3 glycoprotein (Florman and Wassarman, 1985), which lead to aggregation of the sperm receptor and trigger acrosomal exocytosis. The nature of the sperm binding oligosaccharides on ZP3 remains unclear, as are the sperm proteins that bind ZP3 (Florman and Wassarman, 1985; Johnston et al., 1998; Miller et al., 1992; Nagdas et al., 1994; Nishimura et al., 2001; Primakoff and Myles, 2002; Rankin et al., 1998; Wassarman et al., 2001). One candidate that has been extensively studied is β1,4-galactosyltransferase I (GalT I). A wealth of evidence suggests that GalT I functions as a ZP3 receptor and participates in G protein-dependent acrosomal exocytosis following ZP3-mediated GalT I aggregation (Gong et al., 1995; Miller et al., 1992). In this regard, ectopic expression of GalT I on *Xenopus* oocytes leads to specific ZP3 binding and G protein activation. Site-directed mutagenesis of the GalT I cytoplasmic domain prevents ZP3-dependent G protein activation (Shi et al., 2001). Furthermore, overexpression of GalT I on mouse sperm leads to increased ZP3 binding, G protein activation, and accelerated acrosomal exocytosis (Youakim et al., 1994), whereas GalT I deletion leads to a loss of ZP3 binding and a concomitant loss of zona-induced acrosome reactions (Lu and Shur, 1997). Nevertheless, GalT I null sperm are still able to adhere to the egg coat and fertilize the egg, albeit at low efficiency, although they no longer bind ZP3 (Lu and Shur, 1997). This indicates that sperm adhesion to the zona pellucida requires receptors in addition to GalT I and ZP3, consistent with results from others (Rankin et al., 1998, 2003).

Few sperm proteins remain as viable candidates for mediating sperm adhesion to the zona; however, one particularly attractive candidate is p47, a boar sperm surface protein isolated by affinity chromatography on zona pellucida columns (Ensslin et al., 1998). p47 is homologous to a protein secreted from the mammary epithelial of a variety of species, including mouse (referred to as MFG-E8; Stubbs et al., 1990), rat (rAGS; Ogura et al., 1996), bovine (PAS6/7 or lactadherin; Andersen et al., 1997, 2000), and human (BA46; Couto et al., 1996). In vitro assays suggest that p47-like proteins isolated from milk and a similar protein found in vascular endothelium (Penta et al., 1999) facilitate cell adhesion to the extracellular matrix via an RGD motif in their N-terminal EGF domains, as well as by their C-terminal discoidin (or complement F5/8 type C) domains (Andersen et al., 1997, 2000; Fuentes-Prior et al., 2002).

In this study, we examined whether a p47-like protein functions during fertilization in mouse. A p47-homolog was cloned from mouse testis, for which we propose the name SED1 to bring uniformity to the various names assigned to the mammary and endothelial homologs; SED denotes a *s*ecreted protein containing N-terminal Notch-like type II *E*GF repeats and C-terminal *d*iscoidin/F5/8 C domains. Murine SED1 is localized in the Golgi complex of differentiating spermatogenic cells, as well as in the initial segment of the caput epididymis, which together culminate in SED1 being tightly associated with a discrete domain of the sperm plasma membrane overlying the acrosome. Recombinant SED1 binds selectively to the zona pellucida of unfertilized oocytes, and competition assays with recombinant SED1, various SED1 domain constructs, and anti-SED1 antibodies indicate that SED1 participates in sperm-egg adhesion. This was confirmed by creating SED1 null mice by homologous recombination and examining their reproductive phenotype. SED1 null males show greatly reduced

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fertility in vivo, and their sperm are unable to bind to the egg zona pellucida in vitro without apparent effects on sperm morphology, number, acrosomal status, or motility. Models are proposed to account for the mechanism of action for SED1 during initial gamete adhesion.

Results

Production of Recombinant SED1 and Anti-SED1 Antibodies

Mouse *SED1* was cloned from a testis library (EMBL accession number Y11684). The deduced 426 amino acid (aa) open reading frame encodes an N-terminal 22 aa cleavable signal sequence peptide, two Notch-like type II EGF repeats (the second of which contains an RGD motif), and two C-terminal discoidin/C domains (see Figure 2C insert). Recombinant SED1 was expressed in *E. coli* as a $(His)_6$-fusion protein, purified, and used to immunize rabbits and chickens. The resulting anti-SED1 antibodies recognize a single band of ~58 kDa in sperm and testis lysates (not shown), similar to that seen in mouse milk, which is a rich source of the SED1 homolog MFG-E8 (Stubbs et al., 1990) (Figure 1A). Preimmune sera failed to produce any reaction. SED1 expressed in insect cells migrated as expected for the partially glycosylated recombinant protein.

Immunocytochemical Localization of SED1 in the Male Genital Tract

Testis Immunolocalization

SED1 immunoreactivity localizes to the Golgi apparatus of developing spermatogenic cells (Figures 1B and 1C). SED1 immunoreactivity could be attributed to the Golgi, as opposed to the developing acrosome, due to the characteristic morphology of the Golgi that wraps around the developing acrosomal vesicle. The acrosome has a characteristic clear vacuolar appearance containing the acrosomal granule and is devoid of SED1 immunoreactivity. The localization of SED1 to the Golgi suggests that it is secreted to the sperm surface, consistent with the presence of a cleavable signal sequence peptide (Stubbs et al., 1990). There is some SED1 immunoreactivity in what appears to be Sertoli cells, as well as some interstitial cells between the seminiferous tubules (not shown), the functional significance of which is unclear. Parallel studies using antibodies isolated from rabbit serum or chicken eggs produced similar immunolocalization (not shown). Preimmune rabbit (or chicken) immunoglobulins produced only background levels of staining (Figure 1).

Epididymal Immunolocalization

Sperm leave the testis through the efferent ductules and enter the initial segment of the caput epididymis. Here, they are exposed to SED1 secreted from the principle, or secretory, cells of the epididymal epithelium, which are the predominant immunoreactive cells of the male reproductive tract (Figure 1D). Subsequent portions of the epididymal epithelium show low levels of immunoreactivity, although the sperm remain immunoreactive. As sperm exit the epididymis, SED1 immunoreactivity is high in clear cells of the cauda epididymis, which are responsible for absorption of excess secretion products (Robaire and Hermo, 1994). A lower level of immunoreactivity is found associated with the apical surface of the principle cells of the cauda epididymis. As above, chicken immunoglobulins produced similar localization (data not shown). These data suggest that SED1 is secreted into the lumen of the caput epididymis, where it coats sperm, and excess SED1 is removed by the clear cells of the cauda epididymis as sperm enter the vas deferens.

SED1 Localization on the Sperm Surface

On washed, fixed cauda epididymal sperm, SED1 is restricted to the plasma membrane overlying the acrosome (Figure 1E), the area of the sperm surface responsible for initial binding to the zona pellucida (Primakoff and Myles, 2002; Wassarman et al., 2001). Preimmune IgG produced only background levels of fluorescence (not shown). SED1 localization on the plasma membrane was confirmed by incubating live, unfixed sperm with anti-SED1 antiserum in suspension (see Figure 6B).

Biological Activity of SED1

Anti-SED1 IgG and Recombinant SED1 Inhibit Sperm-Egg Binding

The biological activity of SED1 was initially assessed by competitive sperm-egg binding assays. Purified anti-SED1 IgG produced a dose-dependent inhibition of sperm binding to the zona pellucida, relative to controls (Figure 2A). Sperm motility appeared to be unaffected, even at concentrations of 200 μg/ml IgG, nor was there any evidence of antibody-mediated sperm agglutination that could account for the inhibition of sperm-egg binding. Preimmune IgG had no significant effect on sperm-egg binding at concentrations from 50–200 μg/ml. As expected, the original rabbit anti-SED1 antiserum also produced a dose-dependent inhibition of sperm-egg binding, whereas preimmune serum did not (not shown).

To determine if SED1 itself could inhibit sperm binding to the egg, recombinant SED1 was produced in two different expression systems, purified, and added individually to sperm-egg binding assays. Recombinant SED1, expressed either in bacteria as a $(His)_6$-fusion protein or in insect cells as a GST-fusion protein, competitively inhibited sperm binding to the egg coat in a dose-dependent manner (Figure 2B). Pretreatment of either recombinant protein with Proteinase K destroyed its biological activity (not shown). The two recombinant proteins had similar biological activities when normalized for differences in their molecular weight (MW), suggesting that the N-terminal tags, i.e., GST and $(His)_6$, did not contribute to biological activity, nor did any post-translational processing.

SED1 Function Requires a Discoidin/C Domain

The SED1 protein family has two distinct binding motifs that have been implicated in their adhesive function (Andersen et al., 1997, 2000; Fuentes-Prior et al., 2002; Penta et al., 1999). An RGD motif located within the second EGF domain has been suggested to mediate integrin-dependent binding to cells, whereas the discoidin/C domains are thought to bind negatively charged headgroups on membranes as well as to extracellular matrix components. To determine which, if either, of these domains is responsible for SED1 biological activity during gamete adhesion, different $(His)_6$-fusion proteins were tested for activity in sperm-egg binding assays (Figure 2C).

Intact $(His)_6$-SED1 containing both EGF repeats and

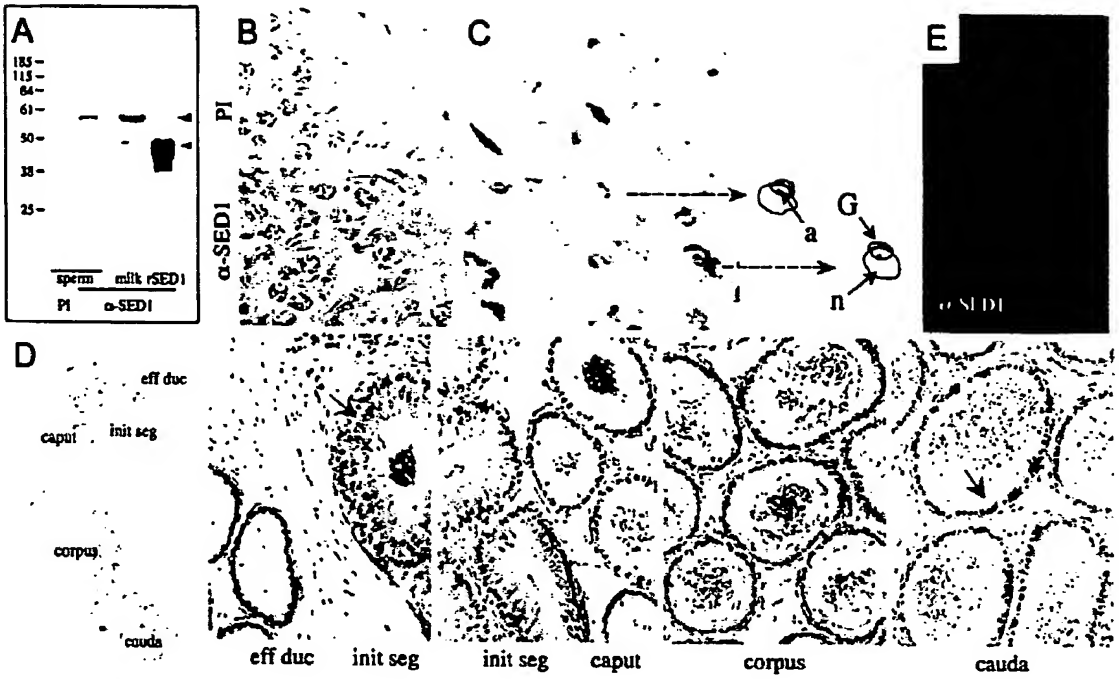


Figure 1. Immunolocalization of SED1 in the Male Reproductive Tract and Sperm

(A) Rabbit antiserum raised against purified recombinant SED1 (α-SED1) specifically recognizes SED1 in sperm. Preimmune serum (PI) fails to show any reactivity. SED1 immunoreactivity in mouse milk is shown as a positive control, as is reactivity to SED1 expressed in insect cells (rSED1). Molecular weight markers are shown on the left. Black arrowhead, mature SED1; gray arrowhead, recombinant SED1.

(B) Testis cross-section incubated with rabbit-α-SED1 and goat-anti-rabbit-HRP/DAB. SED1 is localized to the developing Golgi apparatus in differentiating spermatogenic cells. Original magnification, 600×.

(C) The Golgi (G) wraps around the clear vacuolar acrosome vesicle (a) containing a characteristic acrosomal granule, which is devoid of SED1 immunoreactivity. Tracings of two nuclear (n), acrosome vesicle, and Golgi (G) complexes are presented for orientation. Original magnification, 2500×.

(D) Low-power magnification of the epididymis prepared for anti-SED1 immunocytochemistry; the complete epididymis is represented in two noncontiguous pieces. Representative sections of the epididymis are shown, illustrating the transition between the efferent ductules (eff duc) of the testis, the initial segment of the caput epididymis (init seg), and the caput, corpus, and cauda epididymis. SED1 immunoreactivity is abundantly expressed in the secretory principle cells (arrow) of the initial segment when sperm enter the epididymis, after which the epididymal epithelium is relatively negative for SED1. The secreted SED1 immunoreactivity associates with the sperm throughout their transit through the epididymis. The adsorptive clear cells of the cauda epididymis (arrow), responsible for adsorption of epididymal secretions, show strong SED1 immunoreactivity. Original magnification, 400×.

(E) SED1 is localized to the plasma membrane overlying the acrosome on the anterior sperm head as revealed by α-SED1 and FITC-conjugated goat-anti rabbit IgG. In all instances, preimmune IgG (PI) at the same concentration showed background levels of immunoreactivity (not shown).

both discoidin/C domains (EECC) inhibited sperm-egg binding, as did truncated forms of SED1 (EEC, ECC, EC, CC). Furthermore, the intact and truncated SED1 proteins produced similar levels of inhibition, at constant molar ratios, as long as one discoidin/C domain was present (Figure 2C). Thus, EGF domains are not required for competitive inhibition of sperm-egg binding, implicating the discoidin/C domains in mediating SED1 binding to gamete surfaces. This possibility is consistent with structural and biochemical data, indicating that the discoidin/C domains are able to bind a variety of molecular species, including phospholipid headgroups on membranes, such as on sperm, as well as carbohydrate epitopes in the extracellular matrix, as would be present in the zona pellucida (Fuentes-Prior et al., 2002; Macedo-Ribeiro et al., 1999; Pratt et al., 1999).

Although the above results suggest that the EGF domains do not contribute to competitive inhibition of sperm-egg binding, we directly tested the ability of RGD peptide to competitively inhibit the binding of SED1 to sperm. Recombinant SED1 was conjugated to 0.2 μm fluorescent beads and added to sperm isolated from the caput and cauda (±capacitation) epididymis. RAD peptide was used as control (Buckley et al., 1999). In no instance did RGD peptide produce any specific inhibition of SED1 binding to sperm when assayed at 1–10 mM (Figure 2D). We were unable to assay the biological activity of the isolated EGF domains, since repeated attempts to express the EGF domains as truncated recombinant proteins (EE) failed despite being cloned into three different fusion constructs (i.e., $[His]_6$-EE, GST-EE and Thioredoxin-EE). Nevertheless, the inability of RGD to affect SED1 binding to sperm is consistent with SED1 biological activity residing within the discoidin/C domains, rather than within the EGF repeats.

SED1 Binding to the Zona Pellucida

The results from competitive inhibition assays above are consistent with SED1 participation in sperm-egg binding and implicate the discoidin/C domains in mediating SED1 attachment to gametes. We asked if full-

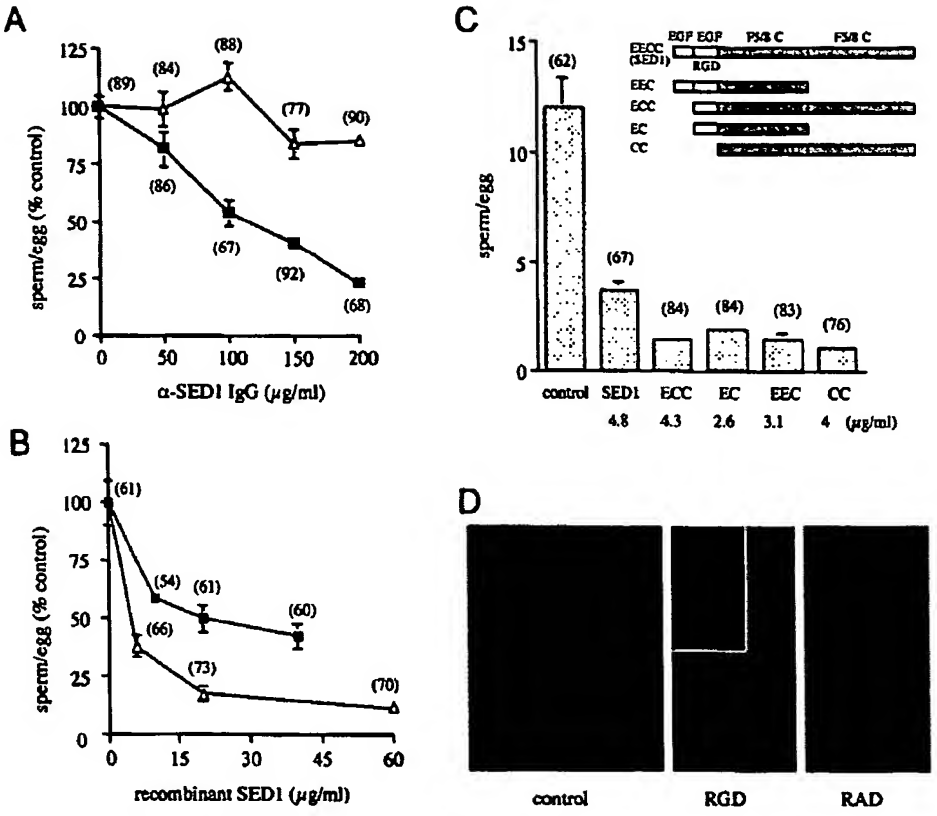


Figure 2. SED1 Functions during Sperm-Egg Binding and Requires the Discoidin/C Domains for Biological Activity

(A) α-SED1 IgG inhibits sperm binding to the zona pellucida in a dose-dependent manner; at 200 μg/ml, sperm-zona binding is suppressed by 75%. Preimmune IgG shows no significant effect on sperm binding to the zona. α-SED1 IgG (closed squares); preimmune IgG (open diamonds).

(B) SED1 fusion proteins expressed in bacteria or insect cells competitively inhibit sperm binding to the zona pellucida in a dose-dependent manner. The two recombinant proteins demonstrate similar biological activity when normalized for MW (data not shown). $(His)_6$-SED1 fusion protein expressed in *E. coli* (open diamonds); GST-SED1 fusion protein expressed in insect cells (closed squares).

(C) Sperm binding to the zona pellucida is competitively inhibited by full-length $(His)_6$-SED1 fusion protein (SED1) and SED1 truncated fusion proteins that contain a discoidin/C domain. The mature SED1 protein is illustrated devoid of its 22 aa N-terminal signal sequence peptide (aa 23–426). The second EGF domain contains an RGD motif. Truncated constructs containing two EGF domains and one F5/8 C domain (aa 23–266, EEC), one EGF domain and both F5/8 C domains (aa 62–426, ECC), the second EGF and one F5/8 C domain (aa 62–266, EC), or both F5/8 C domains (aa 110–426, CC) are illustrated. (A–C) The number of oocytes assayed under each condition is given in parentheses. Control binding represents the number of sperm bound to the zona in the absence of competitive reagents. Error bars = ±SEM.

(D) Fluorescent beads (0.2 μm) conjugated with SED1 bind to the dorsal anterior aspect of the sperm plasma membrane, similar to the distribution of endogenous SED1 (Figure 2D). The presence of 1–10 mM RGD fails to affect SED1 binding to sperm, relative to control peptide (RAD), indicating that SED1 association with sperm is integrin-independent (incubations with 1 mM peptide shown).

length SED1, as well as SED1 truncated proteins, can bind directly to the zona pellucida by assaying (1) the binding of SED1-conjugated beads to intact zona pellucida, and (2) the binding of recombinant SED1 to distinct zona pellucida glycoproteins resolved by SDS-PAGE.

SED1-conjugated 1 μm fluorescent beads were added to droplets containing three sources of zona pellucida: zona pellucida fragments isolated from ovaries; intact, unfertilized ovulated oocytes; and two-cell embryos. Any nonadherent beads were removed by washing. SED1-conjugated fluorescent beads bound directly to the zona pellucida of unfertilized oocytes, as well as to zona fragments isolated from ovaries (not shown), but not to the zona pellucida of fertilized two-cell embryos (Figure 3A). Beads conjugated with truncated forms of SED1 also bound to the oocyte zona pellucida, with constructs containing two discoidin/C domains showing higher binding than did constructs with only one discoidin/C domain (compare EECC and ECC versus EEC and EC, Figure 3B). However, the nature of the assay prevented strict quantification of the binding affinities for the different truncated constructs. In any event, these results further implicate the discoidin/C domains in mediating attachment to the gamete surfaces. Consistent with this, the addition of RGD or RAD peptides had no effect on SED1 binding to unfertilized oocytes (not shown). As expected, WGA beads bound to unfertilized zona pellucida similar to SED1 beads, whereas control beads bound at background levels. Controls included unconjugated beads, as well as beads conjugated with *Ulex europaeus* agglutinin-1 (UEA1) lectin, BSA, or cytochrome C (data shown for UEA1-lectin, Figure 4C).

To determine which of the three zona glycoproteins could serve as the putative ligand for SED1, ovarian

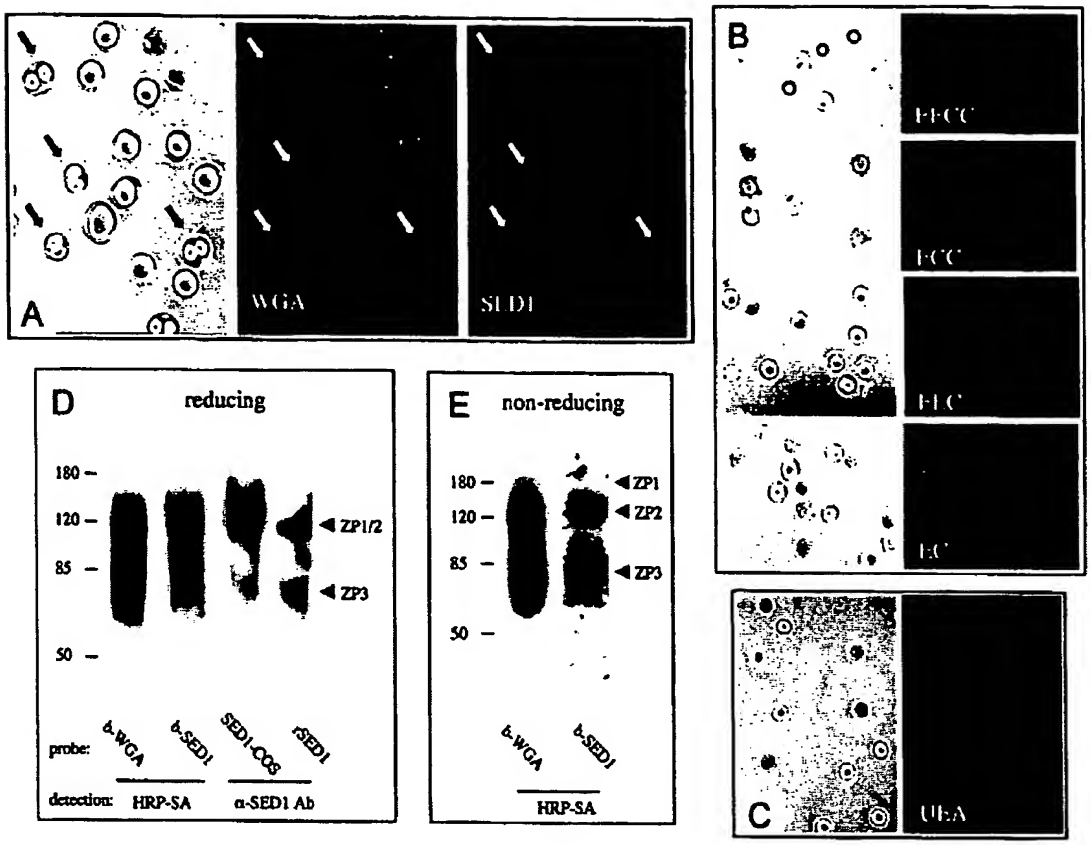


Figure 3. SED1 Binds to the Zona Pellucida of Unfertilized, but Not Fertilized, Oocytes

(A) SED1-conjugated fluorescent beads (1 μm) were added to droplets containing ovulated oocytes and two-cell embryos under conditions identical to sperm-egg binding assays. Unbound beads were removed by washing. The binding of WGA-conjugated beads served as a positive control. SED1 beads readily adhere to the zona of unfertilized oocytes, but not to the zona of fertilized two-cell embryos (arrows). Only a subset of beads is within the focal plane.

(B) Beads conjugated with various truncated SED1 constructs (see Figure 2B) also show specific binding to unfertilized oocytes, although constructs containing both C domains (C1C2) appear to bind more than do constructs with only the C1 domain.

(C) UEA-I-conjugated beads do not bind, consistent with the lack of this epitope on the zona pellucida (Aviles et al., 1997).

(D) When resolved under reducing conditions, ZP1 and ZP2 migrate to a molecular mass of 120 kDa, whereas ZP3 migrates to 80 kDa, as detected by biotin-WGA (b-WGA) and Streptavidin-HRP (HRP-SA). When an identical blot is probed with biotinylated $(His)_6$-SED1 (b-SED1), SED1 binds to the area corresponding to ZP1/ZP2 and to ZP3 . Similarly, when ZP blots were probed with SED1 expressed either in COS-7 cells (SED1-COS) or in bacteria (rSED1) and detected with α-SED1 antibody, binding to ZP1/ZP2 and ZP3 is seen.

(E) Under nonreducing conditions, ZP1, ZP2, and ZP3 resolve at molecular masses of approximately 180, 120, and 80 kDa, respectively, as detected with b-WGA. Recombinant $(His)_6$-SED1 (b-SED1) specifically bound ZP2 and ZP3, but not ZP1.

zona pellucida was purified, resolved by SDS-PAGE, blotted onto PVDF membranes, and incubated with recombinant SED1. When zona glycoproteins were resolved under reducing conditions, SED1 bound to glycoproteins comigrating as ZP1/ZP2 as well as ZP3 (Figure 3D). Similar results were achieved using either biotinylated $(His)_6$-SED1 (b-SED1) detected by Streptavidin-HRP/ECL or recombinant $(His)_6$-SED1 (rSED1) detected by rabbit-anti-SED1 IgG and HRP-conjugated secondary antibodies (Figure 3D). To avoid misleading interpretations due to the use of a bacterially expressed SED1, SED1 was expressed in COS-7 cells, which release budded membrane vesicles that are highly enriched for SED1 (Oshima et al., 2002). Similar to that seen with bacterially expressed SED1, COS-expressed SED1 (SED1-COS) bound ZP1/ZP2 and ZP3 under reducing conditions (Figure 3D).

To distinguish between the ZP1 and ZP2 glycoproteins that migrate similarly under reducing conditions, zona glycoproteins were resolved under nonreducing conditions and probed with biotinylated $(His)_6$-SED1. Under nonreducing conditions, SED1 bound to ZP2 and ZP3. There was no reaction with ZP1 (Figure 3E). SED1 binding to zona glycoproteins could also be demonstrated by performing the reciprocal incubation, i.e., adding solubilized murine zona pellucida to SED1 previously blotted onto PVDF membranes (not shown).

Eliminating SED1 by Homologous Recombination Reduces Male Fertility In Vivo and Sperm-Egg Binding In Vitro

The data thus far suggest that SED1 is localized to the sperm surface where it participates in binding to the zona pellucida of unfertilized oocytes. To test SED1

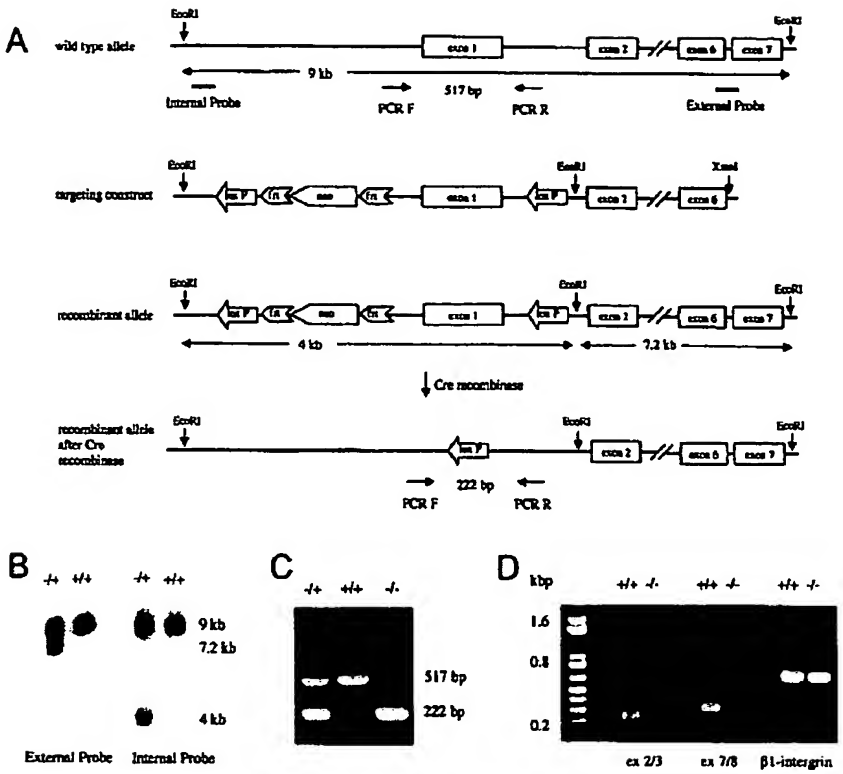


Figure 4. Creation of SED1 Null Mice

(A) A 9kb EcoRI fragment of the wild-type *SED1* allele is illustrated containing exons 1, 2, 6, and 7. The targeting construct contains a neo selection cassette flanked by flip recombinase sites (frt), and lox P sites are inserted 5' of the neo cassette and 3' of exon 1, producing a new EcoRI restriction site.

(B) The recombinant allele can be identified by Southern analysis following EcoRI digestion using external and internal probes (A). Targeted ES clones were transfected with Cre recombinase to excise the neo cassette, adjacent frt sites, and exon 1 encoding the translation start site and signal peptide sequence.

(C) PCR analysis detects a 517 bp product amplified from the wild-type allele and a 222 bp product amplified from the targeted allele.

(D) RT-PCR analysis of testicular RNA illustrates the absence of *SED1* transcripts, using primers spanning the EGF repeats (exons 2 and 3) as well as the discoidin/C domains (exons 7 and 8). As a control, β1 integrin expression appears normal in SED1$^{-/-}$ mice.

function more directly, the effects of targeted mutations in SED1 were analyzed on fertility and sperm-egg binding in vitro.

Creation of SED1 Null Mice

Two independent lines of SED1 null mice were created as described in the Experimental Procedures (Figure 4). The deletion of the *SED1* transcript in homozygous null mice was confirmed by RT-PCR of testicular cDNA using primers specific for the EGF domains (exons 2/3) and the discoidin/C domains (exons 7/8); primers specific for β1-integrin served as control (Figure 4D). Loss of the SED1 protein was confirmed by both immunoblotting and by indirect immunofluorescence of live sperm (Figures 6A and 6B).

SED1 Null Males Are Subfertile

Matings between sexually mature SED1 null littermates produced litter sizes (3.3 ± 0.53 pups/litter, 62 litters) significantly smaller ($p < 0.001$) than from control matings (+/+ matings: 8.5 ± 0.40 pups/litter, 24 litters; +/− matings: 9.3 ± 0.35 pups/litter, 40 litters; +/+ × +/− matings: 9.6 ± 0.40 pups/litter, 10 litters, not shown) The reduced litter size from SED1 null matings could be attributed directly to the reduced fertility of the SED1 null males rather than reduced female fertility; i.e., matings between SED1 null males and wild-type females produced small litters (4.0 ± 0.41 pups/litter, 31 litters, $p < 0.001$), whereas SED1 null females mated to wild-type males generated near normal litter sizes (7.7 ± 0.44 pups/litter, 26 litters, $p = 0.23$). Both independent lines of SED1 null mice produced nearly identical average litter sizes, which could be attributed to the male parent (data not shown) (Figure 5A).

Litter sizes resulting from the 16 control breeding pairs (all combinations of +/+ and +/− adults) were generally similar in size. In marked contrast, the litter sizes from the SED1 null matings varied considerably among the breeding pairs, ranging from no pups produced in 5 of 20 breeding pairs to litter sizes approaching the lower limit of normal variation. The variable phenotype in SED1 null males is not unprecedented (Nayernia et al., 2002; Pearse et al., 1997) and may reflect the segregation of genetic modifiers on the mosaic genetic background resulting from the incorporation of 129 ES cells into C57Bl/6 blastocysts (Wolfer et al., 2002). Testing this

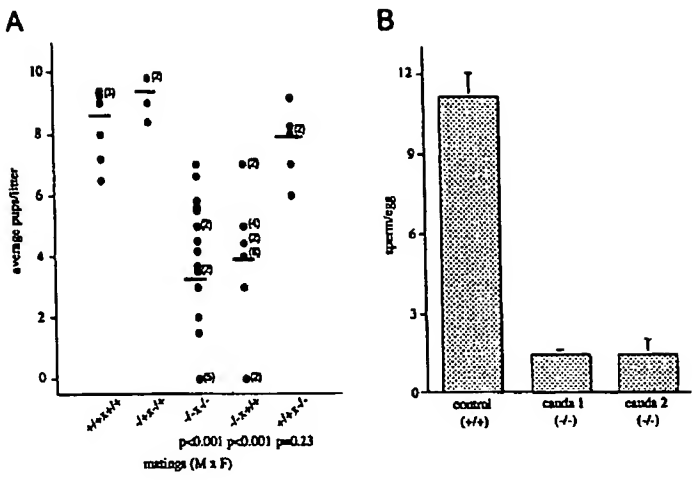


Figure 5. SED1 Null Males Show Reduced Fertility In Vivo and Their Sperm Fail to Bind the Zona Pellucida in Vitro

(A) The frequency distribution of litter sizes resulting from each breeding pair is presented. Each dot represents the average litter size resulting from a single breeding pair (0–20 litters/pair). In instances where breeding pairs produced identical average litter sizes, the number of pairs is given in parentheses. All control breeding pairs produced an average of 8.5–9.3 pups/litter, whereas matings between SED1 null mice produced an average of 3.3 pups/litter. This average reflects wide variability in male fertility, ranging from apparent sterility in five males (i.e., no pups) to litter sizes approaching the lower limit of normal. The reduced litter size in homozygous null matings is due to reduced fertility of the male ($p < 0.001$ relative to control matings) rather than the female, which is near normal ($p = 0.23$ relative to controls).

(B) Sperm isolated from the cauda epididymis of SED1 males (−/−) bind to the zona pellucida at very low levels (1.45 ± 0.56 sperm/egg), as compared to wild-type littermates (+/+) (11.2 ± 0.85 sperm/egg). Error bars = ±SEM.

possibility must await the backcrossing of SED1 null animals onto a congenic background to determine if this results in a more penetrant male phenotype (i.e., higher rates of male sterility). Nevertheless, it is clear that the fertility of SED1 null males is greatly compromised, with some showing complete sterility whereas others show litter sizes still smaller than normal.

Sperm from SED1 Null Males Binds Poorly to the Zona Pellucida

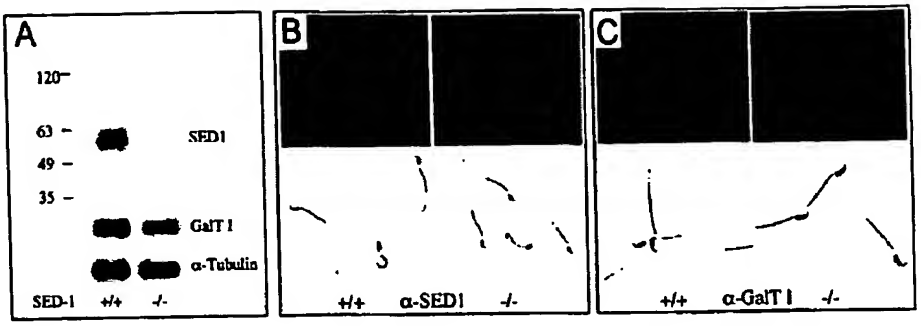
The reduced fertility of SED1 males is consistent with the function of SED1 in sperm-egg binding. We therefore compared the ability of sperm from wild-type and SED1 null males to bind the zona pellucida in vitro. Sperm isolated from each SED1 null cauda epididymis was assayed separately, rather than pooled as is normally done with wild-type sperm, in case there were differences between the two cauda. SED1 null sperm bound to the zona pellucida at levels close to background, i.e., 1.45 ± 0.56 sperm/egg versus 11.2 ± 0.85 sperm/egg using wild-type sperm (Figure 5B). Sperm isolated from both SED1 null cauda epididymides displayed similar levels of decreased sperm/egg binding. The reduced binding of SED1 null sperm to the egg coat was not the secondary result of defective sperm morphology; number; percent motility; or the level of basal, spontaneous or ionophore-induced acrosome reactions, all of which were indistinguishable between normal and SED1 null sperm (Figure 6). Collectively, these results show that sperm from SED1 null mice are relatively unable to bind to the zona pellucida, both confirming its role in normal sperm-egg adhesion and offering a mechanism to account for the reduced male fertility in vivo.

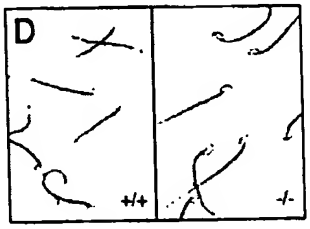
We have previously reported that GalT I on the sperm surface functions as a receptor for the zona pellucida glycoprotein ZP3 (Miller et al., 1992), an observation that is supported by both gain-of-function and loss-of-function GalT I mutations (Lu and Shur, 1997; Shi et al., 2001; Youakim et al., 1994). However, sperm from GalT I null males still bind to the zona pellucida, although they fail to bind ZP3 or undergo zona-induced acrosomal exocytosis (Lu and Shur, 1997). This led us to search for novel sperm receptors for the egg coat, such as SED1. Since it has been reported that elimination of some sperm proteins (i.e., the ADAMS) can have unanticipated effects on the expression of unrelated sperm components (Nishimura et al., 2001), we determined if the elimination of SED1 had any unanticipated effects on GalT1 expression or function. This was shown not to be the case, since SED1 null sperm express GalT I normally by both immunoblot analysis and indirect immunofluorescence of live sperm (Figures 6A and 6C). In addition, the binding of GalT1 null sperm to the zona is inhibited by anti-SED1 IgG (33% of control binding at 200 μg/ml versus 91% for preimmune IgG), recombinant SED1 (27% of control at 6 μg/ml), and the EEC truncated protein (21% of control at 3.8 μg/ml). Thus, the expression and function of GalT I and SED1 are independent of one another, consistent with their having distinct roles during sperm-egg binding.

Discussion

SED1 As a Gamete Adhesin

The results presented here support the involvement of SED1 in mouse gamete recognition. SED1 is homologous to a small group of bimotif secreted proteins containing N-terminal EGF domains and C-terminal discoidin/complement domains (Andersen et al., 1997, 2000; Couto et al., 1996; Ogura et al., 1996; Stubbs et al., 1990). SED1 is expressed in the Golgi complex of spermatogenic cells and is likely secreted onto the maturing sperm surface. The most abundant expression of SED1 in the male reproductive tract occurs in the initial segment of the epididymis, where sperm are exposed to high levels of secreted SED1. The presence of SED1 immunoreactivity in the adsorptive clear cells of the cauda epididymis suggests that excess SED1 is removed from the sperm environment before they leave the epididymis. On mature sperm, SED1 expression is confined to the sperm plasma membrane overlying the acrosome, the known location for sperm binding to the egg coat. Recombinant SED1, expressed in either bac-





phenotype/genotype	+/+	-/-
# sperm (x10⁶)/male	5.8 ±0.49 (9)	5.9 ±0.24 (9)
% motile sperm	59.3 ± 2.47 (6)	59.7 ±3.02 (6)
% basal AR-sperm	3.6 ±0.35 (5)	4.5 ±0.73 (5)
% spontaneous AR-sperm/60 min	21.5 ±1.68 (5)	20.5 ±2.86 (5)
fold increase in A23187-induced AR-sperm	1.7, 2.0 (2)	2.0, 2.2 (2)

Figure 6. Sperm from SED1 Null Males Show No Apparent Defects in a Variety of Other Parameters

Cauda epididymal sperm from wild-type (+/+) and SED1 null (−/−) males were assayed for the expression of SED1 and GalT I by immunoblotting (A) and indirect immunofluorescence of live, unfixed sperm (B and C). As expected, SED1 null sperm are devoid of SED1 but express GalT I normally. Blots were probed with antibodies against α-tubulin to assess protein loading. Fluorescence images are paired with the corresponding phase-contrast image. Cauda epididymal sperm from wild-type and SED1 null males show grossly normal morphology, as seen in phase contrast images (B and C) as well as after staining for acrosomal status (D). Wild-type and SED1 null males produced similar numbers of cauda epididymal sperm, which showed similar levels of motility, as well as the extent of basal, spontaneous, and ionophore-induced acrosome reactions (table, number of males assayed given in parentheses).

teria or insect cells, competitively inhibits sperm-egg binding, as do anti-SED1 antibodies. The biological activity of SED1 requires the discoidin/C domains, which appear to be responsible for SED1 attachment to the sperm membrane and to the zona pellucida matrix. Direct binding of SED1 to the zona pellucida of unfertilized, but not fertilized, eggs is consistent with its role in sperm binding, and immunoblot overlay assays suggest that SED1 recognizes the ZP2 and ZP3 glycoprotein families. Given the ability of discoidin/C domains to bind complex carbohydrates matrices (Fuentes-Prior et al., 2002; Reitherman et al., 1975), it is most likely that SED1 is binding to the carbohydrates residues of ZP2 and ZP3, although this awaits further analysis.

It is somewhat surprising that SED1 should interact in vitro with both the ZP2 and ZP3 glycoproteins, since it is believed that only ZP3 functions as the initial binding ligand for sperm. ZP2 has been suggested to function as a binding ligand for acrosome-reacted sperm (Wassarman et al., 2001). It is unknown if the binding of recombinant SED1 to zona glycoproteins resolved by SDS-PAGE accurately reflects the true binding specificity of native SED1 to the intact zona or if differences in binding affinities for these distinct zona glycoproteins or their oligosaccharide chains would be revealed in this type of assay. Alternatively, sperm may recognize the "supramolecular" organization of the zona, rather than individual zona glycoproteins, as recently suggested (Rankin et al., 2003). All of these possibilities require further study.

Independent of its zona binding specificity, the function of SED1 during fertilization was directly tested by creating SED1 null mice and analyzing their fertility. All SED1 null males produced smaller litters than controls, although the average litter size per male varied considerably, ranging from no pups born (apparent sterility) to litter sizes approaching the lower limit of normal. This variability is most likely due to genetic factors segregating on the mosaic 129/B6 background as was shown to be the case for at least two other targeted mutations in sperm. Mice bearing mutations in either the *Sprm-1* transcription factor (Pearse et al., 1997) or the *Smcp* mitochondrial protein (Nayernia et al., 2002) lead to male sterility only when inbred on the 129/Sv line. Despite the variability in male fertility in vivo, sperm from all SED1 null males tested showed near background levels of binding to the zona pellucida in vitro, consistent with a role for SED1 in sperm-egg binding.

SED1 and GalT I Function Independently

SED1 expression and function is independent of GalT I, a previously identified sperm receptor for the zona pellucida glycoprotein, ZP3 (Miller et al., 1992). GalT I is expressed normally on SED1 null sperm, and GalT I null sperm are equally sensitive to inhibition by SED1 antagonists, as are wild-type sperm. Analysis of GalT I function suggests that it acts specifically as a signaling component for ZP3-dependent acrosomal exocytosis (Gong et al., 1995; Lu and Shur, 1997; Shi et al., 2001; Youakim et al., 1994), since GalT I null sperm are unable

to bind ZP3 in solution or undergo zona-induced acrosomal exocytosis, although they still bind to the egg coat. In contrast to GalT I, SED1 appears to function during the initial adhesion between the sperm and egg coat. Consequently, these results suggest the simple working model that sperm binding to the egg coat requires at least two distinct binding events: initial adhesion between the gametes facilitated by SED1 and a subsequent interaction between GalT I (and possibly other sperm components) and ZP3 oligosaccharides, leading to GalT I aggregation and acrosomal exocytosis. If sperm-egg binding involves at least two distinct molecular interactions, it is unclear why reagents against either one (i.e., GalT I or SED1) are able to inhibit sperm-egg binding when assayed individually. Possibly, these two molecular complexes functionally interact or some of these inhibitory reagents may sterically interfere with adjacent sperm surface components. Alternatively, the overall process of sperm-egg binding may result from the summation of temporally-specific stages, each defined by a unique molecular interaction, that are currently below the resolution of in vitro sperm-egg binding assays. In an analogous manner, a multiplicity of molecular interactions facilitating gamete adhesion is reminiscent of the sequential requirement of selectins and integrins during lymphocyte interactions with the vascular endothelium (Worthylake and Burridge, 2001).

SED1 Functional Domains

The involvement of SED1 in the initial adhesion of sperm to the extracellular coat of the egg is consistent with studies of two SED1 homologs, lactadherin (also known as PAS 6/7) and Del1, which are thought to act as bimotif adhesive proteins that facilitate cell adhesion to the extracellular matrix (Andersen et al., 1997; 2000; Penta et al., 1999). In particular, the discoidin/C domains are found in a wide variety of proteins and are thought to mediate protein binding to cell membranes and to extracellular matrix components (Fuentes-Prior et al., 2002). Recent structural analysis of these domains indicates that they fold into an eight-stranded antiparallel β barrel from which two (or three) hairpin spikes project that present polypeptide-specific residues for interacting with various binding surfaces. For example, the spikes found in the C2 domain of complement possess hydrophobic residues that intercalate into the lipid bilayer, surrounded by a ring of charged residues that dock with phosphatidylserine headgroups (Macedo-Ribeiro et al., 1999; Pratt et al., 1999). The F5/8 C domains in "discoidin," the galactose binding lectin from *Dictyostelium*, display a completely different binding specificity in that spikes projecting from the discoidin β barrel coordinate binding to galactose residues rather than to membrane phospholipids (Fuentes-Prior et al., 2002; Reitherman et al., 1975). Similar to that seen in the discoidin lectin, the chitobiase enzyme from *Arthrobacter* utilizes a discoidin/C domain to attach to glycans terminating in sialic acid, galactose, and *N*-acetylglucosamine (Lonhienne et al., 2001). In all instances, hypervariable spikes project from the eight-stranded β barrel core and are thought to determine the specificity of binding to membranes and to the extracellular matrix (Fuentes-Prior et al., 2002).

It is, therefore, not surprising that the discoidin/C domains appear to be required for SED1 biological activity, based upon the ability of discoidin/C-containing constructs to competitively inhibit sperm-egg binding as well as to bind directly to the zona pellucida. There is no evidence in this system that SED1 function involves RGD recognition by integrins. Although there have been some preliminary reports of integrins on human sperm membranes (Fusi et al., 1996; Trubner et al., 1997), their presence has not been documented in mouse. Furthermore, the α subunit (i.e., α_v) reported to bind the RGD domain in lactadherin (Andersen et al., 1997) and Del1 (Penta et al., 1999) is not present on the surface of fresh, ejaculated, or capacitated human sperm (Fusi et al., 1996). More directly, the addition of RGD had no specific effect on SED1 association with sperm. Consequently, the data are consistent with a working model in which the discoidin/C domains mediate interactions with both the sperm membrane and the egg coat. In this regard, both of the SED1 discoidin/C domains have the amino acids required to form the two hairpin loops that present site-specific residues for docking to membranes and/or matrices (Fuentes-Prior et al., 2002).

Models for SED1 Function during Gamete Adhesion

Some working models for SED1 function can be developed from the results presented here, from SED1 sequence analysis, and from studies of SED1 homologs in other systems. Given that the discoidin/C domains can account for SED1 binding to both the sperm membrane and the zona pellucida, the simplest model proposes that SED1 functions as a monomer, whereby the two discoidin/C domains of a single SED1 polypeptide are sufficient to bind sperm to the zona pellucida (Figure 7A). Since constructs containing both C domains (C1C2) show increased binding to the zona relative to constructs with only the C1 domain (Figure 3), it is possible that the C2 domain has greater affinity for the zona, whereas the C1 domain mediates attachment to the sperm membrane.

Alternatively, SED1 may mediate sperm-egg binding as a dimer, or oligomer, as has been illustrated for the epithelial cell adhesion molecule, Ep-CAM (Figure 7B). Ep-CAM mediates adhesions by homophilic binding between the two Notch-like EGF repeats; the first EGF domain appears responsible for interactions with Ep-CAM on apposing cells, whereas the second EGF domain is thought to mediate lateral oligomerization of Ep-CAMs within the membrane (Balzar et al., 2001). This is particularly relevant to understanding SED1 function, since both EGF repeats in SED1 contain classic Notch-like consensus sequences (Balzar et al., 2001), and only two EGF repeats are required for Notch-like protein-protein binding (Balzar et al., 2001; Lawrence et al., 2000) (Figure 7C). The first EGF repeat of SED1 contains the Notch O-glycosylation consensus sequence, suggesting that O-glycosylation may regulate SED1 dimer (or oligomer) formation, analogous to its regulation of Notch-ligand binding (Haltiwanger, 2002).

In either scenario, one discoidin/C domain (e.g., C1) is postulated to mediate SED1 attachment to sperm, whereas a second discoidin/C domain (e.g., C2), either

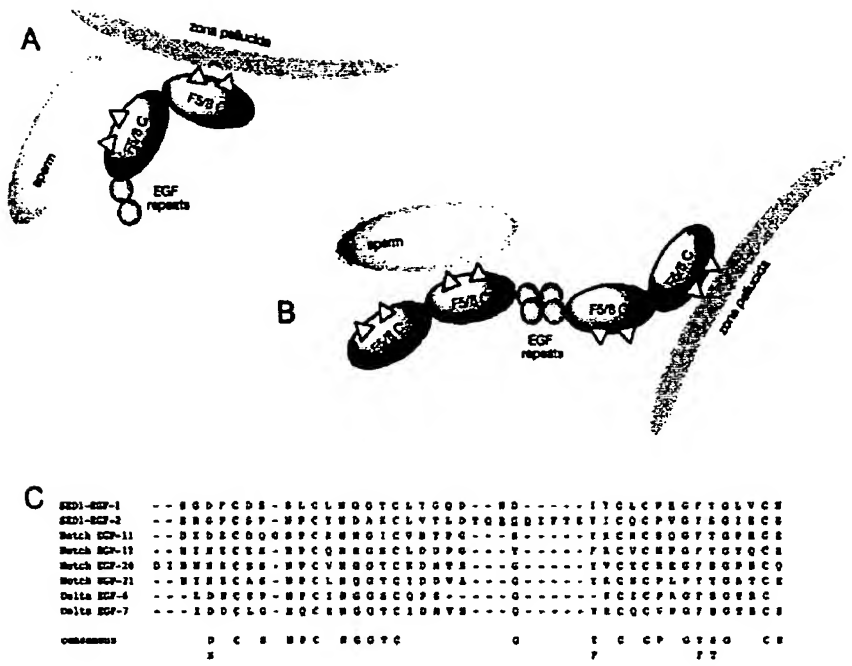


Figure 7. Models for SED1 Function during Sperm-Egg Binding

(A) SED1 binding to gamete surfaces appears to be mediated by the discoidin/C domains rather than the EGF repeats. Consequently, the simplest model for SED1 function suggests that the two discoidin/C domains of one SED1 monomer binds to both the sperm membrane (via C1) and the zona pellucida (via C2). The binding specificity of each discoidin/C domain for either membranes or matrix components is thought to reside in the hypervariable spikes (triangles) that emerge from the disulfide-bonded barrel core, as determined by crystallographic studies (Macedo-Ribeiro et al., 1999; Pratt et al., 1999).

(B) Alternatively, SED1 may function during sperm-egg binding as a dimer, or oligomer, due to anti-parallel pairing of the EGF repeats, similar to that reported for the EGF domains of Ep-CAM (Balzar et al., 2001). Consistent with this, the EGF repeats of SED1 contain the Notch consensus sequences required for Notch-Delta pairing (Balzar et al., 2001; Lawrence et al., 2000).

(C) SED1 EGF repeats 1 and 2 are aligned with a variety of Notch-family EGF domains. The conserved six cysteines are in gray, residues that match the Notch EGF consensus (consensus) sequence are in yellow, conserved residues are in orange, and the O-glycosylation consensus sequence is in blue (Haltiwanger, 2002).

within the SED1 monomer or dimer partner, is exposed for association with the zona pellucida (Figure 7B). Recent crystallographic data suggests that the exposed, or unoccupied, discoidin/C domain remains in a cryptic, or closed, conformation until presentation to its binding surface (i.e., zona pellucida), which stabilizes the active, or open, binding conformation (Fuentes-Prior et al., 2002).

Neither EGF repeats or discoidin/C domains have been implicated in mammalian fertilization previously, although a sperm surface protein containing EGF repeats is thought to be required for sperm-egg binding in *C. elegans* (Singson et al., 1998). Therefore, the identification of SED1 proposes a previously unappreciated mechanism for mammalian gamete recognition, which takes advantage of highly conserved protein motifs that facilitate binding to a wide range of cell surfaces and extracellular matrices.

Experimental Procedures

Cloning and Expression of Mouse SED1

Murine *SED1* cDNA was cloned from testis RNA by RT-PCR using primers based upon published data (Enssfin et al., 1998). SED1 and truncated forms of SED1 were expressed in *E. coli* with an N-terminal $(His)_6$-tag (Invitrogen). Cell were lysed, insoluble material solubilized in resolving buffer (8 M urea, 30 mM phosphate, 50 mM Tris-HCl [pH 8.1], and 5 mM imidazole), and subjected to affinity chromatography on Ni^{2+}-Sepharose (Invitrogen). Recombinant protein was selectively eluted from the column using resolving buffer supplemented with 250 mM imidazole and renatured by repetitive dialysis against PBS supplemented with 0.3 mM DTT.

Full-length SED1 with a N-terminal GST tag was constructed using the pMelBac baculovirus transfer vector (Invitrogen). Upon cotransfection with Bac-N-Blue DNA (Invitrogen), viral DNA containing SED1 was isolated, and high-titer viral stocks were produced. High five cells were infected and incubated for 65 hr at 27°C, and the cell supernatant was assayed for SED1 expression by Western blotting. For GST-SED1 purification, the cell supernatant was adjusted to pH 7.4 and 1 M urea (final concentration) with subsequent addition of glutathione-Sepharose (Pharmacia). GST-SED1 was eluted from glutathione-Sepharose using 100 mM phosphate (pH 8.6), 50 mM glutathione, 1 M urea, and 1 mM DTT, followed by extensive dialysis in PBS with 0.3 mM DTT.

For expression in COS-7 cells, SED1 was amplified by RT-PCR and cloned into the pcDNA3.1D/V5-His-Topo mammalian expression vector (Invitrogen). COS-7 cells were transfected with FuGENE (Roche) in serum-free DMEM according to the manufacturer's protocol. SED1 enriched membrane vesicles secreted into the culture media were purified using differential centrifugation (Oshima et al., 2002).

Recombinant SED1 and truncated forms of SED1 were biotinyl-

ated using EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce) according to the manufacturer's protocol.

Antibody Production and Western Blot Analysis

Affinity-purified $(His)_6$-tagged SED1 was subjected to preparative SDS-PAGE. The gel was stained with 100 mM KCl at 0°C (Nelles and Bamburg, 1976), and the band representing SED1 was removed and served as antigen for the immunization of rabbits and chickens. Reactivity of preimmune and immune sera was determined using ELISA. Purification of anti-SED1 IgG was performed using an IgG Purification Kit (Pierce).

For Western blot analysis, cauda epididymal sperm were washed, pelleted, solubilized in 8 M urea, and the soluble sperm proteins collected after centrifugation. SED1-expressing SF9 cells were washed free of medium, sonicated ,and centrifuged to remove insoluble material. Mouse milk was directly solubilized in sample buffer. In all instances, soluble proteins were resolved on 10% polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore). The membranes were blocked in TBST (150 mM NaCl, 50 mM Tris/HCl [pH 7.5], and 0.1% Tween 20) containing 2% BSA for one hour at 25°C. After incubation with anti-SED1 or anti-GalT I (Youakim et al., 1994) antibody and rinsing, the bound IgG was detected with goat anti-rabbit-HRP (Amersham) and developed using ECL+plus (Amersham). Protein loading was assayed by probing blots with monoclonal antibodies against α-tubulin (Sigma: clone B-5-1-2).

Immunohistochemistry

Testis and epididymis were isolated from CD-1 mice, fixed overnight in Bouin's solution, and paraffin-embedded. Sections (4 μm) were subjected to microwave "antigen retrieval" for two times at 6 min full power with 1 min cooling period, in 10 mM sodium citrate (pH 6) (Janssen et al., 1994). Sections were cooled, blocked, and processed for immunocytochemistry using 1:100 primary antibody, 1:1,000 HRP-conjugated goat-anti-rabbit (Amersham), and "liquid DAB-black substrate kit" (Zymed).

Cauda epididymal sperm were collected in dmKBRT (Lu and Shur, 1997; Youakim et al., 1994), filtered (3-35/27 Nitex, Sefar America), washed three times in PBS, and mounted on polylysine-coated slides. Sperm were fixed at −20°C in methanol for 10 min, rinsed, and blocked for 1 hr in PBS containing 5% normal goat serum (PBS/NGS). Slides were incubated with rabbit anti-SED1 sera (1:100 dilution) in PBS/NGS for 1 hr at 25°C, washed, and incubated with FITC labeled goat anti-rabbit antibodies (1:2,000) (Zymed) for 45 min at 25°C. After washing, the slides were mounted in PBS with 70% glycerol and examined under a Nikon E800 microscope. For analysis of live sperm, 100 μl of washed sperm (5×10^6 sperm/ml) in PBS, 0.25% BSA were mixed with antiserum against SED1, GalT I, or preimmune serum (1:50 final dilution) and incubated for 30 min at 25°C. The sperm suspension was washed free of excess antiserum by layering onto 1 ml of PBS, 3% BSA, and centrifuged at 600 × g for 5 min at 25°C. The sperm suspension was mixed with Fab fragments of goat anti-rabbit (1:1000 final, Alexa Fluor 594, Molecular Probes), or goat anti-rabbit-FITC (Zymed), for 30 min at 25°C in the dark, and washed as described above. The sperm were fixed in 1% paraformaldehyde for 10 min and viewed under a Nikon E800 microscope. Controls were prepared by substituting the primary antibody with preimmune serum and nonspecific rabbit IgGs.

Sperm Capacitation and Binding to the Egg Coat

Sperm capacitation and egg binding assays were performed as previously reported (Lu and Shur, 1997). For analysis of acrosomal status, capacitated sperm were fixed in 2% paraformaldehyde, stained with Coomassie, and viewed under the microscope as described (Larson and Miller, 1999). To induce the acrosome reaction, A23187 (10 μM final, Sigma) was added to capacitated sperm and incubated an additional 10 min before fixation and Coomassie staining. For egg binding assays, eggs from 8-week-old superovulated females were freed from their cumulus cells by hyaluronidase treatment (0.2%, 10 min, 25°C), washed in dmKBRT, and added in groups of 20 eggs to 40 μl drops dmKBRT supplemented with competing agents under paraffin oil. Three to five two-cell embryos flushed from oviducts of superovulated females were included in every drop as negative controls. 10 μl sperm solution was added to the egg-containing drops and binding was allowed to proceed for 30 min at 37°C. Afterwards, sperm-egg complexes were washed free of unbound or loosely bound sperm. Washing was stopped when <3 sperm remained bound to two-cell embryos. Sperm-egg complexes were fixed in 4% formaldehyde, and the number of sperm attached to the egg zona pellucida was determined.

Binding of SED1-Coated Microspheres to Zona Pellucida and Sperm

Neutr-Avidin-labeled fluorescent beads (Fluo-Spheres, Molecular Probes), 1 μm, were coated with biotinylated SED1 proteins as described by the manufacturer. WGA-biotin served as a positive control protein; UEAI-biotin, BSA-biotin, cytochrome C-biotin, and uncoupled Fluo-Spheres were used as negative controls (Aviles et al., 1997). Ovulated oocytes, two-cell embryos, and zona pellucida from ovarian oocytes were isolated as described and combined into 40 μl droplets of dmKBRT. To these droplets, 5–10 μl of protein-coated Fluo-Spheres were added and incubated for 1 hr at 25°C. The suspension was washed with a microbore pipette to remove unbound Fluo-Spheres and subsequently examined under the microscope.

For analysis of SED1 binding to sperm, cauda epididymal sperm was collected in dmKBRT buffer, filtered and washed twice (400 × g, 5 min), and adjusted to 10^3 sperm/μl. To 50 μl sperm suspension, 3 μl of SED1-coated Fluo-Spheres (0.2 μm) and 7 μl of either GRGDNP-peptide (Biomol) or GRADSP control peptide (Buckley et al., 1999) was added, final concentration 0–10 mM, and incubated for 30 min at 25°C. Sperm were washed twice in dmKBRT buffer to remove unbound Fluo-Spheres, mounted, and examined under the microscope.

Purification of Zona Pellucida Glycoproteins for SED1 Blot Overlay Analysis

The purification of zona pellucida glycoproteins was done as published elsewhere (Miller et al., 1992). The proteins were resolved on 10% polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore) as above. The membranes were blocked in TBST containing 2% BSA for 1 hr at 25°C. Subsequently, membranes were incubated with either $(His)_6$-SED1, biotinylated $(His)_6$-SED1, SED1 prepared from COS-7 cells, or biotinylated WGA-lectin (positive control) for 1 hr at 25°C and washed three times for 10 min in TBST, followed by incubation with either rabbit anti-SED1 and goat anti-rabbit-HRP, or Streptavidin-HRP (Zymed). The membranes were washed, developed, and exposed to film as above.

Creation of SED1 Null Mice

Two BAC clones were identified that contained the *SED1* gene by screening a commercial 129/Sv ES cell mouse BAC library (Genome Systems) with PCR primers corresponding to exon 2 and exon 3. A 9 kb EcoRI fragment containing the SED1 coding region was subcloned into the bluescript vector KS+ (Figure 4A). The first loxP site, as well as the neo cassette and two frt sites, were inserted 140 bp upstream of the ATG start signal, leaving 1.45 kb of 5′ sequences. The second loxP site was inserted 160 bp downstream of the signal peptide. At the 3′ end, the target vector was shortened by 1.5 kb. An additional EcoRI site was introduced into the *SED1* gene to create diagnostic restriction sites for identifying the targeted allele (Figure 4A).

The target vector (TVSED1) was linearized and transfected into ES cells. DNA from neomycin-resistant clones was subjected to a restriction digest with EcoRI, separated on an 0.8% agarose gel, blotted onto a nylon membrane, and probed for an homologous recombination event using ^{32}P-dCTP-labeled probes either internal to the region of homology (372 bp Internal Probe) or external (335 bp External Probe) to the targeting construct (Figure 4B). Out of 288 neomycin-resistant ES clones, 13 (4.2%) could be identified as positive for the targeted SED1 allele.

Two independent parental clones positive for the *SED1*-targeted allele (#164, #252) were transfected with Cre recombinase to excise the neo-selection cassette and the exon containing the signal peptide. The genotype of the ES clones was confirmed by PCR analysis

using primers that amplify either a 517 bp fragment from the wild-type allele or a 222 bp fragment from the targeted allele (forward, 5′-CCTCAGGCTGAGGACTGGCAGCGGC; reverse, 5′-GCTGTCAC CGGGTGTCCAGGGTCACC) (Figure 4A). Four ES cell clones were expanded and injected into blastocysts, which were transferred into pseudopregnant females. The blastocyst injections resulted in nine highly chimeric mice, assayed by coat color. The presence of the mutated *SED1* allele was confirmed in two mice from each of the original parental clones. Chimeras were mated to C57Bl/6 wild-type females to create founder mice heterozygous for the *SED1* mutation. Homozygous SED1 null mice were created by heterozygous matings. Genotypes of the resulting mice were determined using the same PCR strategy as used for genotyping ES clones (Figure 4C).

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Accession Numbers

The nucleotide databank accession number for the murine SED1 protein is Y11684.

CLAIMS

We claim:

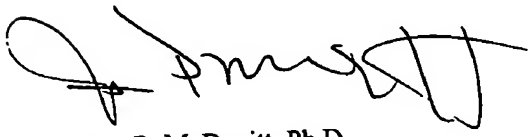
1. A method for enhancing the likelihood of fertilization of an egg in a mammalian species comprising addition of SED1 to a solution containing sperm or an egg.
2. The method of claim 1, wherein SED1 is added to an in vitro mixture of sperm and one or more eggs.
3. A method for enhancing the likelihood of fertilization of an egg in a mammalian species comprising addition of SED1 Functional Protein to a solution containing sperm or an egg.
4. The method of claim 3, wherein SED1 Functional Protein is added to an in vitro mixture of sperm and one or more eggs.
5. The method of claim 4, wherein SED1 Functional Protein is recombinant protein.
6. The method of claim 5, wherein said recombinant SED1 Functional Protein is produced in bacteria or yeast.

7. A method for diagnosing infertility in a male comprising:
   (i) Obtaining sperm from a male
   (ii) Assaying said sperm for the presence of SED1 protein

8. The method of claim 2, wherein the amount of SED is quantified and compared to normal SED1 concentrations in sperm.
9. A method for decreasing the likelihood of pregnancy comprising administering to a female a composition comprising a SED1 variant having a binding site for eggs but lacking a binding site for sperm.
10. A method for decreasing the likelihood of pregnancy comprising administering antibodies to SED1 to a mammal.

SMALL ENTITY STATEMENT

In accordance with 37 C.F.R. 1.27, I assert that Emory University is entitled to small entity status for this application, Emory docket number 03093Prov.



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